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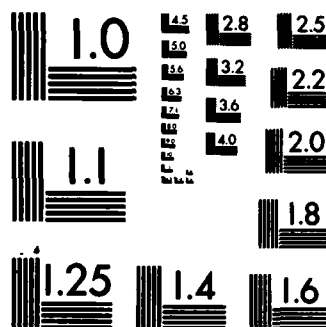
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ELECTROPHORETIC LIGHT SCATTERING:

INSTRUMENTATION AND APPLICATION TO THE MEASUREMENT

OF THE ELECTRIC CHARGE OF HUMAN BLOOD CELLS

by

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An instrumentation system which combines electrophoresis and laser Doppler light scattering velocimetry was used to measure the electrophoretic mobility of human blood cells and platelets. The technique is called Electrophoretic Light Scattering (ELS), and the measurement obtained from it reflects the magnitude of the negative charge on the surface of these cells.

The instrument, built from its constituent components and coupled to a

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Hewlett-Packard data acquisition system, was used for storage, retrieval, and analysis of the electrophoretic spectra. The performance of the instrument using "standard" fixed cells was considered to be excellent.

Subsequently, ELS was used to explore its capabilities in achieving the objectives of this laboratory, especially in deriving physical parameters of predictive value in ascertaining the quality of preserved red blood cells and platelets. It was found that neither liquid storage (up to 30 days) nor reticulocyte enrichment had any effects on the electric charge of erythrocyte populations. Furthermore, neither induced shape alterations such as are known to occur on storage, nor physical conditioning of the donors produced any changes in the charge of the red blood cells. It was, therefore, concluded that measurement of the surface charge of the red blood cells and platelets does not provide a sensitive enough index to be of value in the quality control of blood preservation techniques.

In studies with human granulocytes, there was no correlation between zymosan A activation and surface charge alteration.

1.

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Doppler light scattering velocimetry was used to measure the electro-

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In studies with human granulocytes, there was no correlation between zymosan A activation and surface charge alteration.

A. Electrophoresis

Electrophoresis is the physical process involved in the migration of electrically charged particles in an electric field provided by a uniform d.c. potential.[1] The particle and its ionic environment are known as the electric double layer, and many theories exist about its electrokinetic behavior.[2] The imaginary outer boundary of this layer corresponds to the surface of shear and represents the slippage plane between a particle and its suspending medium. The electrical potential of this plane is called the zeta potential (ζ), and its magnitude can be estimated from the Helmholtz-Smoluchowski[3,4] equation:

$$\zeta = 4\pi\eta u/\epsilon \tag{1}$$

where ζ is the zeta potential (volt), u is the electrophoretic mobility (cm^2/volt sec) and η and ϵ are the viscosity (poise) and dielectric constant, respectively. The electrophoretic mobility is estimated from measurements of electrophoretic velocity v (cm/sec) and electric field strength E (volt/cm):

$$u = v/E \tag{2}$$

The zeta potential is useful in estimating under certain assumptions the electric charge density on the surface of biological cells.

B. Electrophoretic Light Scattering

1. Introduction

In 1971, Ware and Flygare[3] introduced the electrophoretic light scattering (ELS) method for measuring the electrophoretic mobility (u) of macromolecules. The technique combines electrophoresis in free solution and laser Doppler velocimetry. Uzgiris[4] first applied the method to the determination of the electrophoretic velocities of biological cells, and since then a number of investigations have been carried out by ELS (see References 5 and 6 for review). The main advantages of the method over other electrophoretic techniques are that measurements can be made within a few seconds and that no particle concentration gradients are developed because the polarity of the field is alternated after each measurement.

2. Principle and Instrumentation

The principle of ELS and instrumental assembly according to Ware[5,6]

are illustrated in Fig. 1. A coherent, monochromatic and polarized

beam of laser (argon) light is scattered by particles (blood cells)

located in the scattering cell. The particles move perpendicularly

to the beam by the influence of an electric field. A photomultiplier

(PMT) positioned at an angle (ϕ) with respect to the beam, collects

the scattered light from the illuminated particles during electro-

phoresis as synchronized by the sweep trigger. The collected light

is transformed by the PMT into an electrical signal which is then

analyzed by the spectrum analyzer (Digital Real Time Fast Fourier Transform

Analyzer). The spectrum analyzer computes the power spectrum and

accumulates the average successive spectra collected during a series

of electric field pulses. A computer is then used to calculate the

mean frequency shift and peak width of the averaged power spectra.

By knowing the electric field strength, one can calculate the electro-

phoretic mobility of the particle (see A above).

Fig. 2 illustrates the characteristics of the electrophoretic

chamber. The chamber consists of two metal electrodes, each with a

semicircular cutout separated by plastic inserts. To satisfy the

optical requirements of the system, the front and back of the cell

are made of glass. The result of this geometry is a circular sampling

chamber which is divided into halves by plastic inserts which have a

small gap between them. When the current is switched on, a homogeneous

electrical field is formed in the gap area which is used for the electro-

phoresis. When a laser beam is focused in this gap, particles under-

going electrophoresis are illuminated and the resulting scattered light

intensity fluctuations are collected by the PMT (Fig. 3). The Fast

Fourier Transform or Spectrum analyzer determine the shift in frequency

of the monochromatic light due to the Doppler effect (Fig. 3). Because

the diffusion coefficient (Brownian motion) of the cells is very small,

only the electrophoretic motion contributes to the Doppler shift. This

is to say, the Doppler shift is proportional to the electrophoretic

velocity (v) of the particle which depends on the electrophoretic

mobility (u) and applied electric field strength E. The observed

frequency shift (Δv) is given by:

$$\Delta v = (nuE/\lambda)\sin(\phi/2) \tag{3}$$

where n is the refractive index of the liquid medium, λ is the vacuum wavelength of the laser light, and ϕ is the scattering angle. E and u are defined above. From this relationship, the electrophoretic mobility of the particles can be estimated.

C. Experimental Set-Up for ELS

1. Instrumental Set-Up at NBRL

The electrophoretic light scattering apparatus was constructed in this laboratory as described by Ware.[5,6] The electric mobility spectra are transferred in digital form from the ELS apparatus to the Hewlett-Packard 9435 data acquisition system for analysis. The system formats the data appropriately and stores them on a floppy disk for subsequent retrieval. All the necessary computer software to perform this operation were developed in this laboratory.

2. Electrophoresis Buffer

All electrophoretic measurements were made in an iso-osmotic sucrose buffer solution which had a pH of 7.4 and an ionic strength of

0.013 M. The solution was made by diluting 1 part of phosphate-buffered

saline (PBS) with 14 parts of 0.029 M sucrose and was stored at 4°C.

Prior to use the buffer was filtered (0.45 μ filter) to remove particulate

matter and was allowed to equilibrate to within 2°C of the temperature

of the electrophoretic cell.

3. Instrument Standardization

A human red cell suspension (fixed in an iso-osmotic sucrose

buffer solution containing 1% glutaraldehyde and stored at 4 C) was

used to standardize the instrument. This is necessary to correct for

the following: a) changes in the current density in the gap due to

cross-sectional area changes; the area changes are due to the flow of

grease sealer in the area of the gap; b) changes in electrode resistance

which varies with the age of the electrode; c) variances in buffer

conductivity between buffer preparations, and d) electro-osmosis due

to reduced effectiveness of the interior coating of the chamber. Therefore,

the electrophoretic results were normalized to the electrophoretic

mobility of the "standard" to achieve the required precision in

reporting mobilities.

4. Resolution

The resolution obtained by the instrument can be checked

by using a mixture of glutaraldehyde-fixed human and rabbit red blood

cells (RBC). Human RBC exhibit significantly higher electrophoretic

mobility than rabbit cells, so the two populations should be

completely separated. The resolution can be estimated from the mean

difference in mobility of the two populations and the peak width.

Resolution above 1 indicates complete separation. Typical spectra

of such resolution experiments are shown in Fig. 4 and indicate the

excellent performance of the instrument.

D. Applications of ELS to the Study of Human Blood Cells

1. Liquid Storage Study of Red Blood Cells

An exploratory study was conducted to determine whether a

relationship exists between the time of liquid storage (at 4°C) of

human red blood cells and alterations in electrophoretic mobility. It

was hypothesized that damage to the RBC membrane might be reflected on

the electric charge of the cell surface and, therefore, such a relationship might serve as a useful quality control parameter.

Human blood for this study was collected in citrate-phosphate-dextrose (CPD) from healthy volunteers and was stored as such at $4^{\circ}C$ for 30 days. During this period the electrophoretic mobility of the red blood cells was measured after dilution of the red blood cells with the iso-osmotic sucrose buffer solution to a concentration of 6×10^4 cells/ml. The total elapsed time from the dilution of the blood to the completion of all measurements was kept to within 15 minutes.

Fig. 5 illustrates the results of this study. The erythrocytes stored in the liquid state as whole blood at $4^{\circ}C$ exhibited a gradual and small decrease in relative electrophoretic mobility as a function of storage time. However, this change was considered to be of insignificant magnitude to be used as a quality control parameter.

2. <u>Electrophoretic Mobility of Reticulocyte-Enriched Red Blood Cell Populations</u>

These experiments were performed to ascertain if there is a relationship between electrophoretic mobility and red blood cell age.

Reticulocyte-enriched red blood cells were isolated by using either the Fenwal CS-3000 Cell Separator or the low density phthalate fractionation method, and their mobility was measured after appropriate dilution. There was no correlation between reticulocyte count in RBC populations and electrophoretic mobility (Fig. 6). Likewise, no consistent trend in mobility changes could be demonstrated between whole blood and Fenwal CS-3000-isolated red blood cells (Fig. 7). The variation in the mobility was greater in the latter than in the whole blood RBC, but this difference was attributed to the methodology of isolation.

3. <u>Electrophoretic Mobility of RBC Collected from Human Subjects</u> <u>After Physical Conditioning</u>

Whole blood was obtained from 14 healthy volunteers prior to initiation of a three-month period of physical conditioning. The samples were frozen with 40% w/v glycerol and stored at $-80^{\circ}C$. After three months of physical conditioning additional samples were drawn from the volunteers and were frozen in the same manner. Both the pre- and post-

frozen blood samples were thawed and washed on the same day, and

measurements were made of the electrophoretic mobility of the red blood

cells. No consistent relationship in electrophoretic mobility was seen

between the pre- and post-physical conditioning red blood cell samples

(Fig. 8).

4. Red Blood Cell Shape and Surface Charge

During liquid storage of blood there is a progressive shape

change of red blood cells from discocytes to echinocytes and sphero-

cytes. Since, theoretically, the electrophoretic mobility is

proportional to the ratio of surface charge density to the frictional

coefficient, it was considered desirable to assess the effect of red

blood cell morphology on its electrophoretic mobility. Therefore,

discocytes, echinocytes and spherocytes were prepared from the same

red cell population and then fixed in glutaraldehyde. The assumption

here was that fixation did not alter the surface charge density. The

cells were prepared as follows:

Discocytes: Cells with volumes ranging from 80 μ^3 to 130 μ^3 were

classified as discocytes. The discocytes were prepared by allowing whole blood to equilibrate for 10 minutes in phosphate-buffered saline (PBS) containing 1% glutaraldehyde.

Echinocytes: Cells with volumes less than 80 μ^3 were classified as echinocytes. Preparation was achieved by incubating whole blood for 10 minutes in a 1.6% NaCl solution. The cells were then centrifuged and fixed with 1% glutaraldehyde for 10 minutes.

Spherocytes: Cells with volumes over 130 μ^3 were classified as spherocytes. Their preparation involved placing whole blood in 40% w/v glycerol and then transferring the red cells to a PBS solution containing 1% glutaraldehyde.

Fig. 9 illustrates that there was no significant change in electro-phoretic mobility among discocytes, echinocytes and spherocytes. Thus, the cellular morphology and volume changes produced did not significantly alter the charge density of the cells.

5. <u>Zymosan A Activation of Human Granulocytes and Surface Electric</u>
<u>Charge</u>

A study was performed to attempt to quantify the alteration of granulocyte surface charge with zymosan A activation. Two methods of granulocyte collection were involved in this study.

Method 1: a) Dextran (6% in saline) sedimentation of RBC's.

b) Removal of plasma and centrifugation at 1500 g for 10 minutes in a Ficoll-Hypaque density gradient (1.120 and 1.077) at room temperature.

c) Removal of granulocytes from the interface and washing with modified Eagles medium (MEM). Collection by centrifugation at 150 g for 5 minutes.

Method 2: a) Dextran (6% in saline) sedimentation of RBC's.

b) Centrifugation of the plasma at 215 g for 12 minutes at $4^{o}C$.

c) Lysis of contaminating RBC's with 0.2% saline for 20 seconds; reestablishment of isotonicity with the addition of an equal amount of 1.6% saline.

d) Washing in buffer solution.

Fig 10 illustrates that the mean change in surface charge due to activation was -1.40%.

E. Conclusions

A reliable instrumentation system and reproducible methodology were set up for the measurement of the electrophoretic mobility of blood cells.

Under various experimental conditions the electrophoretic mobility of red blood cells was measured. These conditions included: (a) liquid storage of blood at 4 C for up to 30 days; (b) reticulocyte enrichment; (c) three-month physical conditioning of the blood donors; and (d) alteration of the shape of the red blood cell. No useful correlation could be demonstrated between electrophoretic mobility and the variable function of the above experimental conditions.

In addition, no correlation could be demonstrated between mobility and zymosan A activation of human granulocytes.

From the aforementioned, there seems to be little value in using ELS to obtain quality control parameters in the preservation of red blood

cells. However, the instrumentation set-up can be easily modified to

examine the effects of other fields, e.g., magnetic, for future studies.

FIGURE 1

Block diagram of the electrophoretic laser light scattering (ELS)

apparatus. The sample is placed in the "scattering cell" and it is

illuminated by a focused laser beam K_0. The light is scattered (K_s) by

the moving particles, and it is collected by a photomultiplier (PMT) at

a particular angle ϕ . The sweep trigger synchronizes the application

of the electric field from the power supply with the data collection.

The spectrum analyzer (Digital Real Time Fast Fourier Analyzer) calculates

the power spectrum from the photomultiplier signal and accumulates and

averages successive spectra collected during respective electric field

pulses. A computer is used to analyze the averaged spectra and to calculate

the mean frequency shift and peak width which leads to the determination

of the electrophoretic mobility.

FIGURE 1

ARGON LASER
SP 164/02

Lens

t_0

WARE'S SCATTERING CELL

t_{S1}

Lens

LIGHT TRAP

PMT
RCAC31024

PMT POWER SUPPLY
PAR 1107

AMPLIFIER
PAR 113

ELECTRIC FIELD GENERATOR
HP 6186C

SWEEP TRIGGER

REAL TIME SPECTRUM ANALYZER AND DIGITAL AVERAGER
PAR 4513

COMPUTER
HP 9835

POWER

$\Delta\nu$

$\Delta\nu$ (Hz)
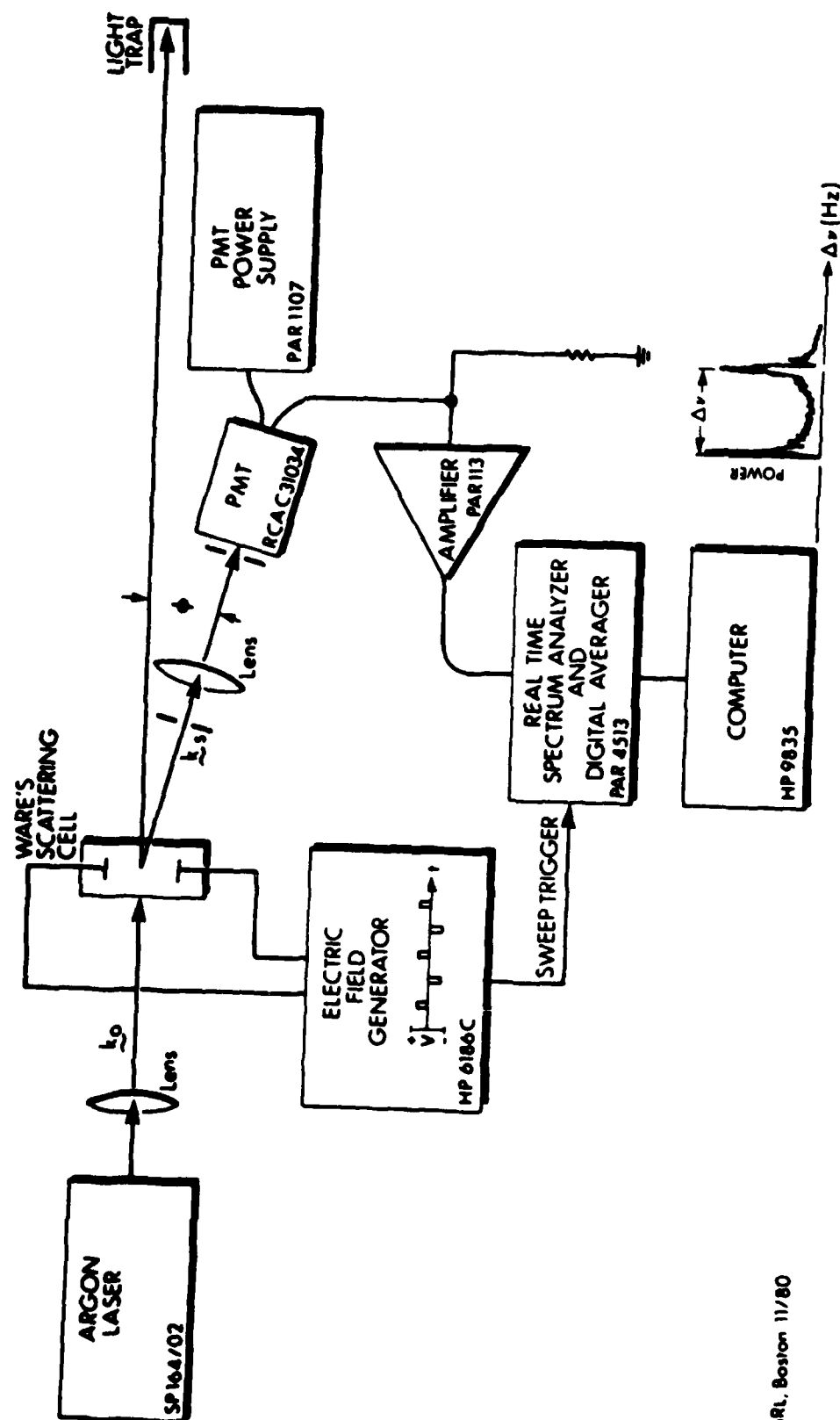
NBRL, Boston 11/80

FIGURE 2

Schematic diagram of the Ware electrophoretic chamber which consists

of a cylindrical optical cell bearing concentric electrodes and cooled by

circulation of fluid. The geometry of the electrodes and the plastic

inserts (shown at the upper right) generate a homogenous electric field

in the gap where electrophoresis occurs.

FIGURE 2

THE WARE'S ELECTROPHORETIC CHAMBER

ELECTRIC FIELD MAP FOR THE HEMICYLINDRICAL ELECTRODES SEPARATED BY A PAIR OF DIELECTRIC INSERTS FORMING A NARROW GAP

SCHEMATIC DRAWING OF A RBC SHOWING SOME NEGATIVELY CHARGED SIALIC ACID RESIDUES

FLOW PATTERN OF THE RBC'S UNDER ELECTROPHORESIS WITH INDICATION OF THE CHARGE DISTRIBUTION OF THE DOUBLE LAYER
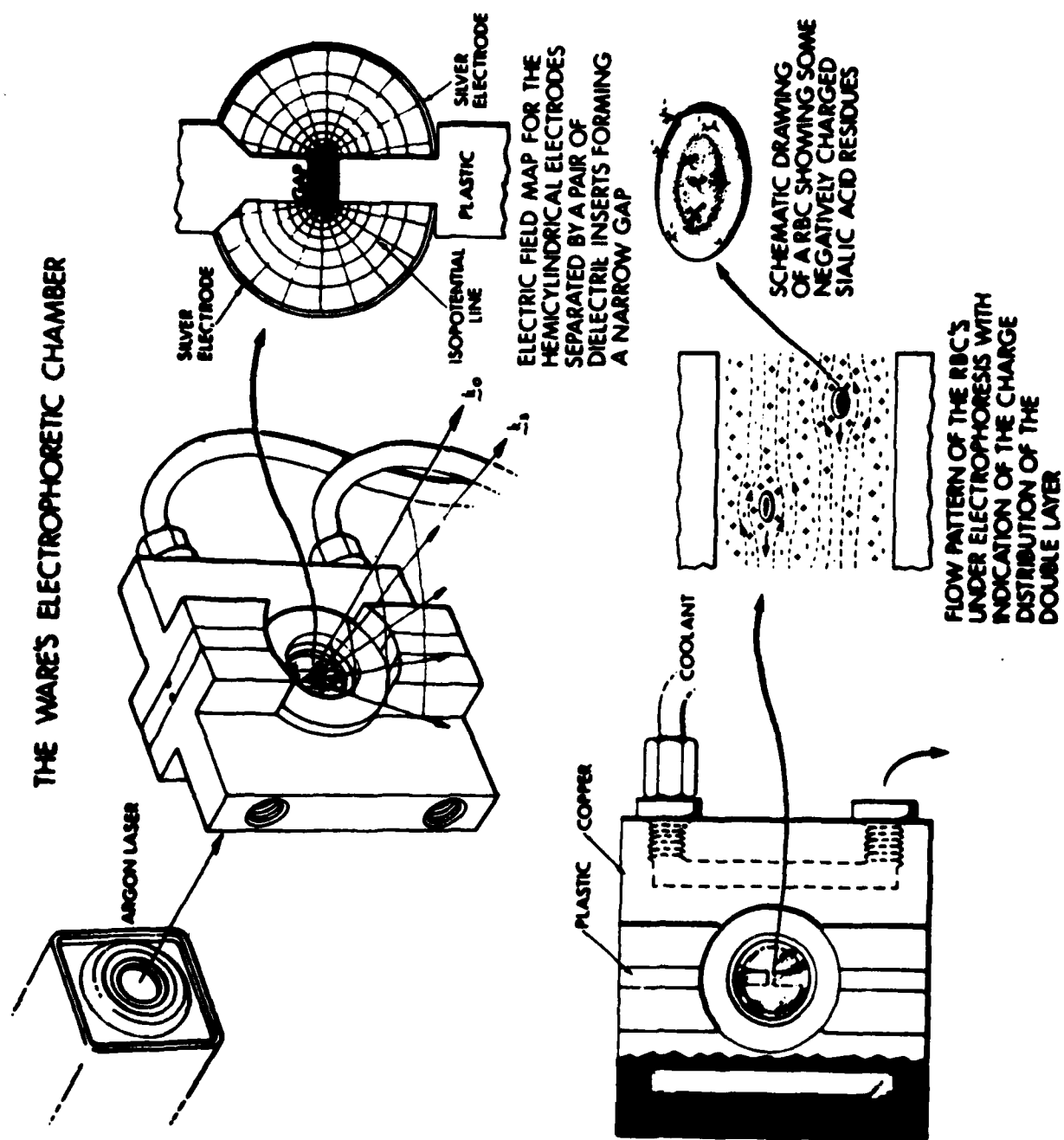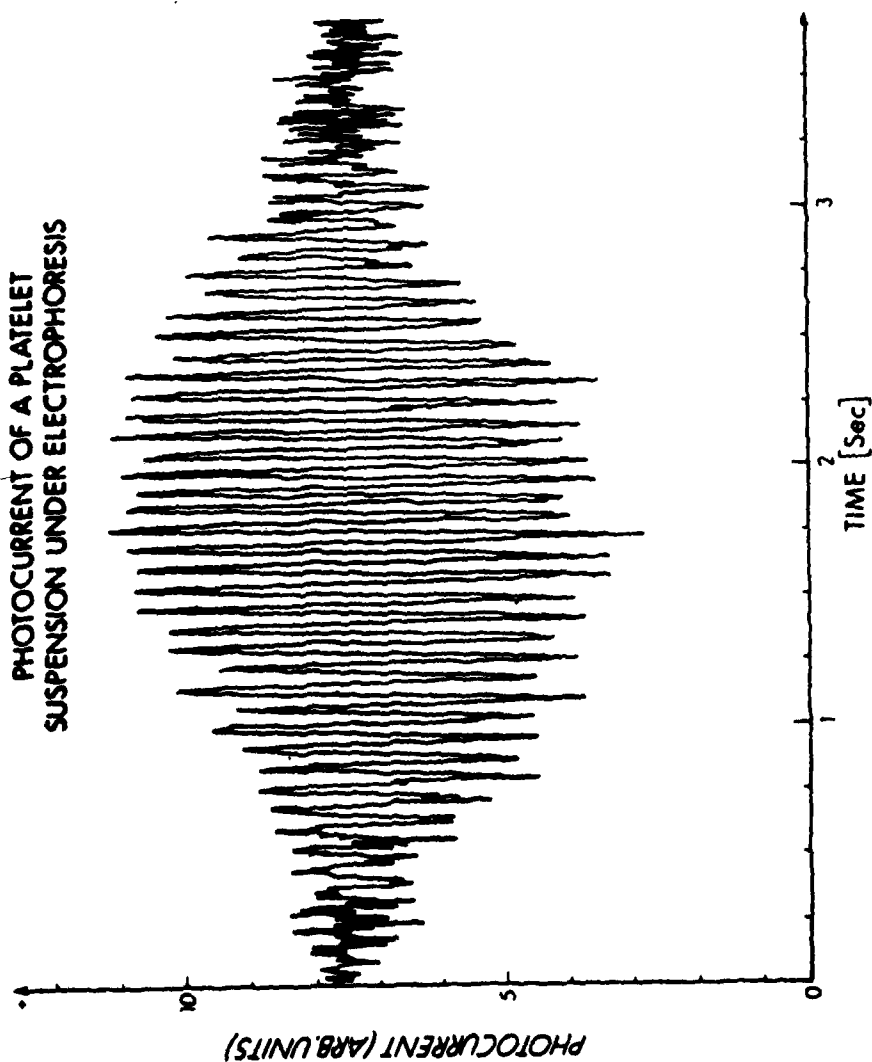
FIGURE 3

Typical photomultiplier current and power spectra of red blood cell

and platelet suspensions subjected to electrophoresis. The scattered

light was collected at an angle ϕ = 7.5 degrees, and the electric field

applied at the gap was 24.5 volt/cm. The characteristic Doppler frequency

shifts (Δv) were 16.5 Hz for fresh unwashed red cells and 13 Hz for fresh

unwashed platelets. (Note the high signal-to-noise ratio of the photo-

current and the sharp and distinct peaks of the collected power spectra.)

For the measurements of Δv (shown on the right hand side of this figure)

only 4 spectra accumulations of 10 seconds were needed.

FIGURE 3

ELECTROPHORETIC LASER LIGHT SCATTERING

PHOTOCURRENT OF A PLATELET
SUSPENSION UNDER ELECTROPHORESIS

POWER SPECTRUM OF THE
LIGHT SCATTERED FROM A
PLATELET AND FROM A RBC
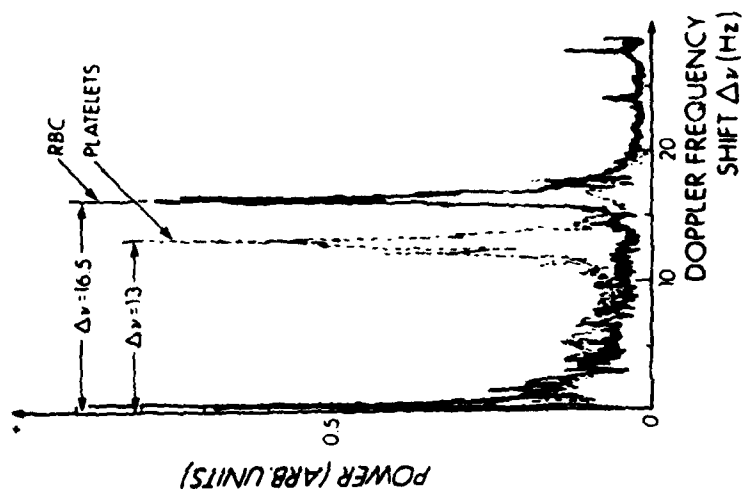SUSPENSION AT 24.5 V/cm

IBRL Boston 11/80

FIGURE 4

Typical electrophoretic spectra of glutaraldehyde-fixed human and

rabbit red blood cells mixed at different proportions: (a) 100% rabbit;

(b) 85% rabbit; (c) 65% rabbit, 35% human; and (d) 50% rabbit, 50% human.

Note the complete separation of the two populations in each case.

FIGURE 4

a
Relative Intensity
Frequency (Hz)
100% Rabbit RBC

b
Relative Intensity
Frequency (Hz)
85% Rabbit RBC
15% Human RBC

c
Relative Intensity
Frequency (Hz)
65% Rabbit RBC
35% Human RBC

d
Relative Intensity
Frequency (Hz)
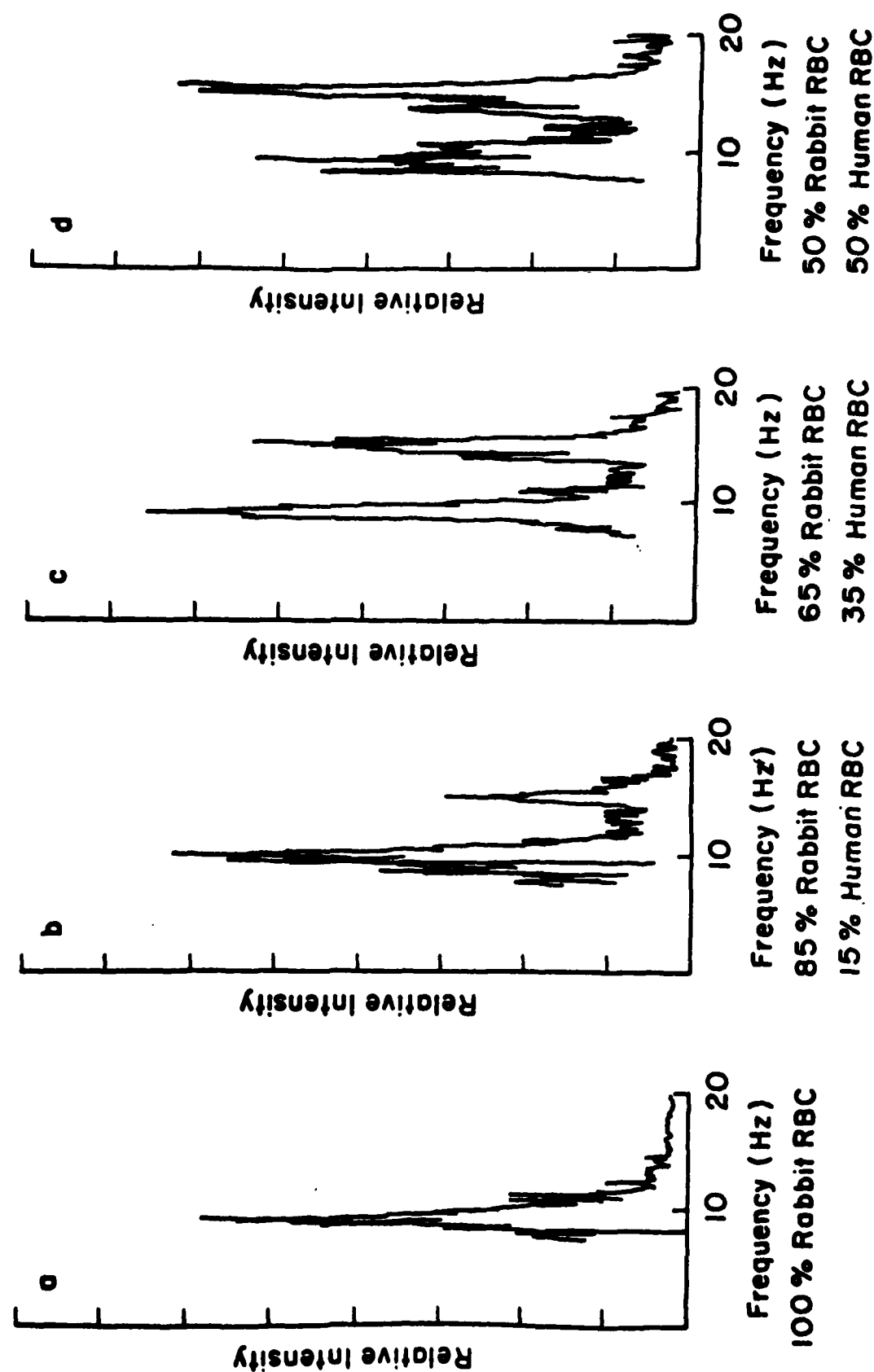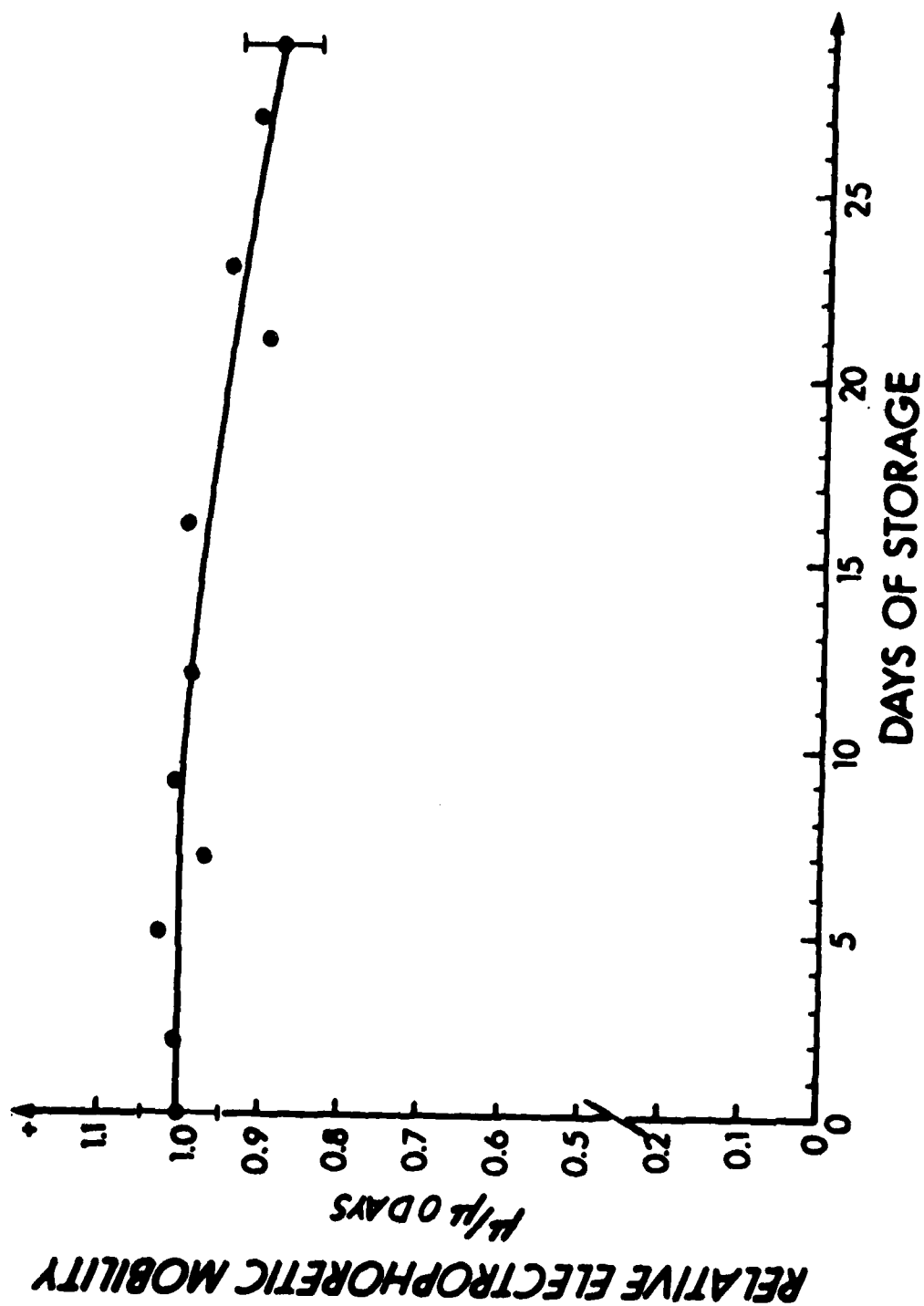50% Rabbit RBC
50% Human RBC

FIGURE 5

Relative electrophoretic mobility (ratio of mobility of stored cells to the mobility of the fresh) of red blood cells stored as whole blood at 4°C as a function of storage time in days.

FIGURE 5

RELATIVE ELECTROPHORETIC MOBILITY
μ/μ 0 DAYS

DAYS OF STORAGE

NBRL, Boston 11/80

FIGURE 6

A sample of "young" (reticulocyte-enriched, up to 20%) red blood cells was obtained from each healthy volunteer using a low density phthalate fractionation procedure along with a control blood sample. The electrophoretic mobility of the "young" red blood cell population was then compared with that of the control red cell population. Data from four studies show no consistent change in electrophoretic mobility between the two samples.
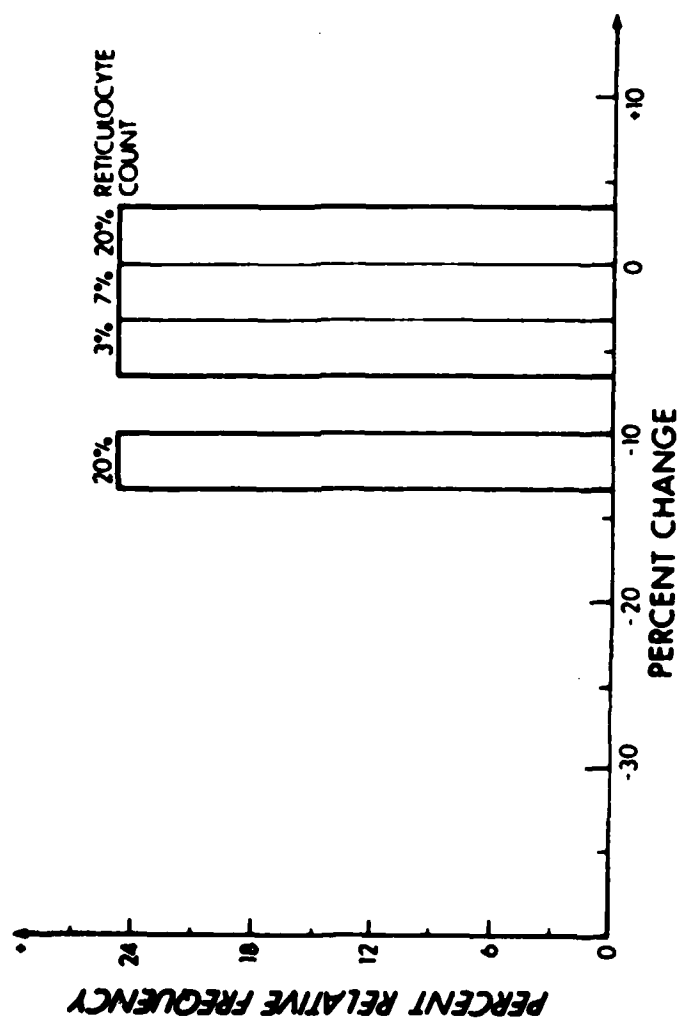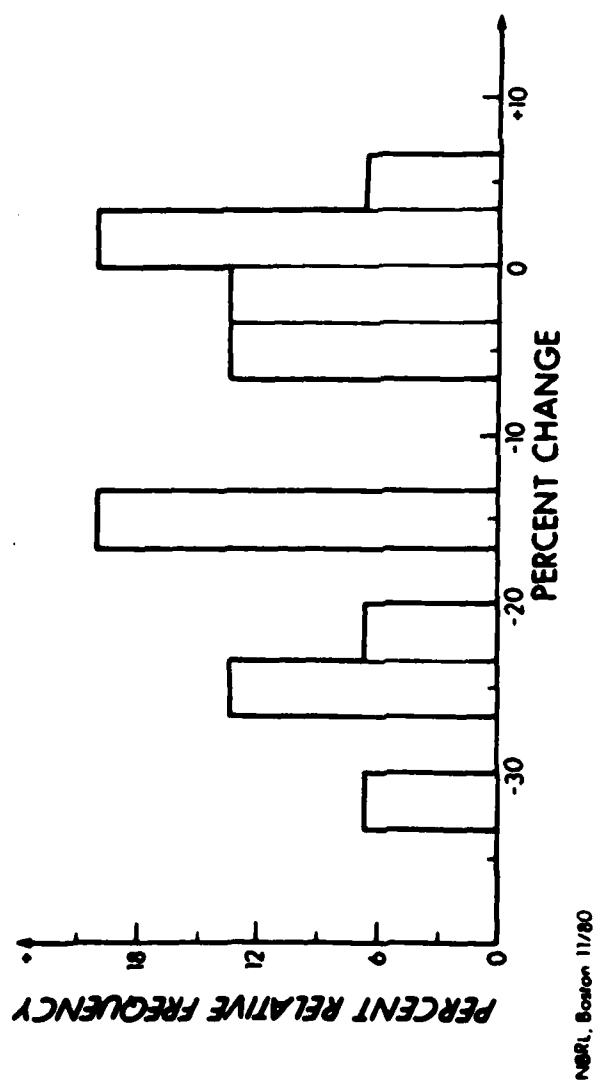
FIGURE 6

FIGURE 7

A sample of "young" (reticulocyte-enriched) red blood cells was obtained from each healthy volunteer using an apheresis procedure with the Fenwal CS-3000 Cell Separator, along with a control blood sample. Data from fifteen studies showed no consistent changes in electrophoretic mobility. The greater mobility variation may be attributed to the methodology of fractionation.

PERCENT CHANGE

PERCENT RELATIVE FREQUENCY

NBRL, Boston 11/80

FIGURE 7

. FIGURE 8

Blood was obtained from healthy volunteers prior to and following

a 3-month period of physical conditioning. The red cells were frozen with

40% w/v glycerol and stored at -80°C. On the day of the study, both the

pre- and the post-exercise blood samples were thawed and washed and the

electrophoretic mobility was measured. The values reported compare the

samples after physical conditioning with the samples prior to physical

conditioning. No consistent trend was observed.

FIGURE 8

PERCENT CHANGE

PERCENT RELATIVE FREQUENCY

NBRL, Boston 11/80

24A
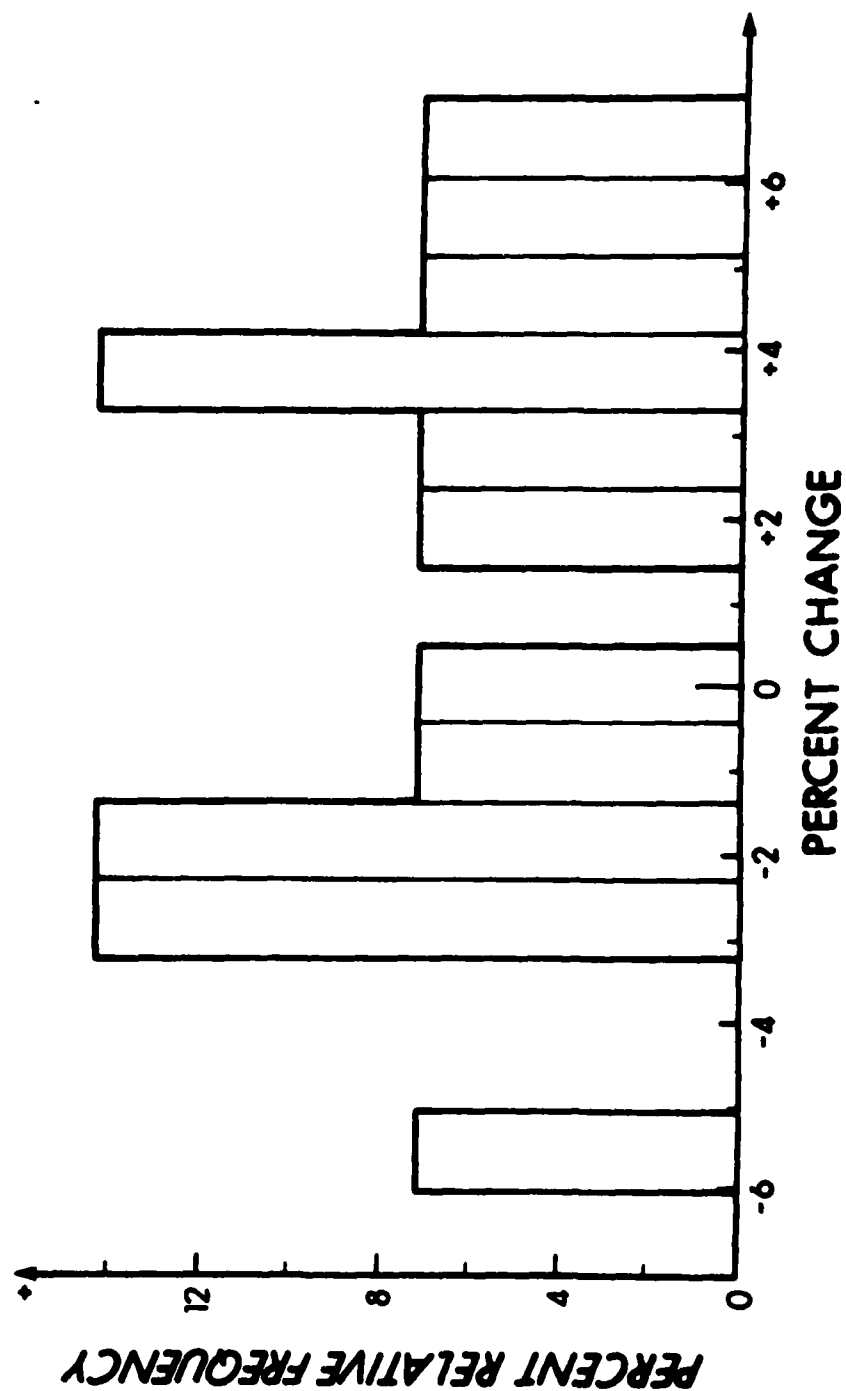
FIGURE 9

Correlation of cellular morphology and electrophoretic mobility (u)
of human red cells. Fresh normal discoidal red blood cells were changed
into echinocytes with volumes of $80\mu^3$ by exposure to a 1.6% NaCl solution
followed by fixation in a 1% glutaraldehyde, and into spheres with volumes
of $130 \mu^3$ by dilution of red cells containing 40% w/v glycerol with a
phosphate-buffered saline solution containing 1% glutaraldehyde. The
relative electrophoretic mobility (ratio of mobility of altered cells to
the mobility of the discoids) in relation to red cell volume is plotted
for each of the three donors. No significant change in relative
electrophoretic mobility was seen with the changes in cellular morphology
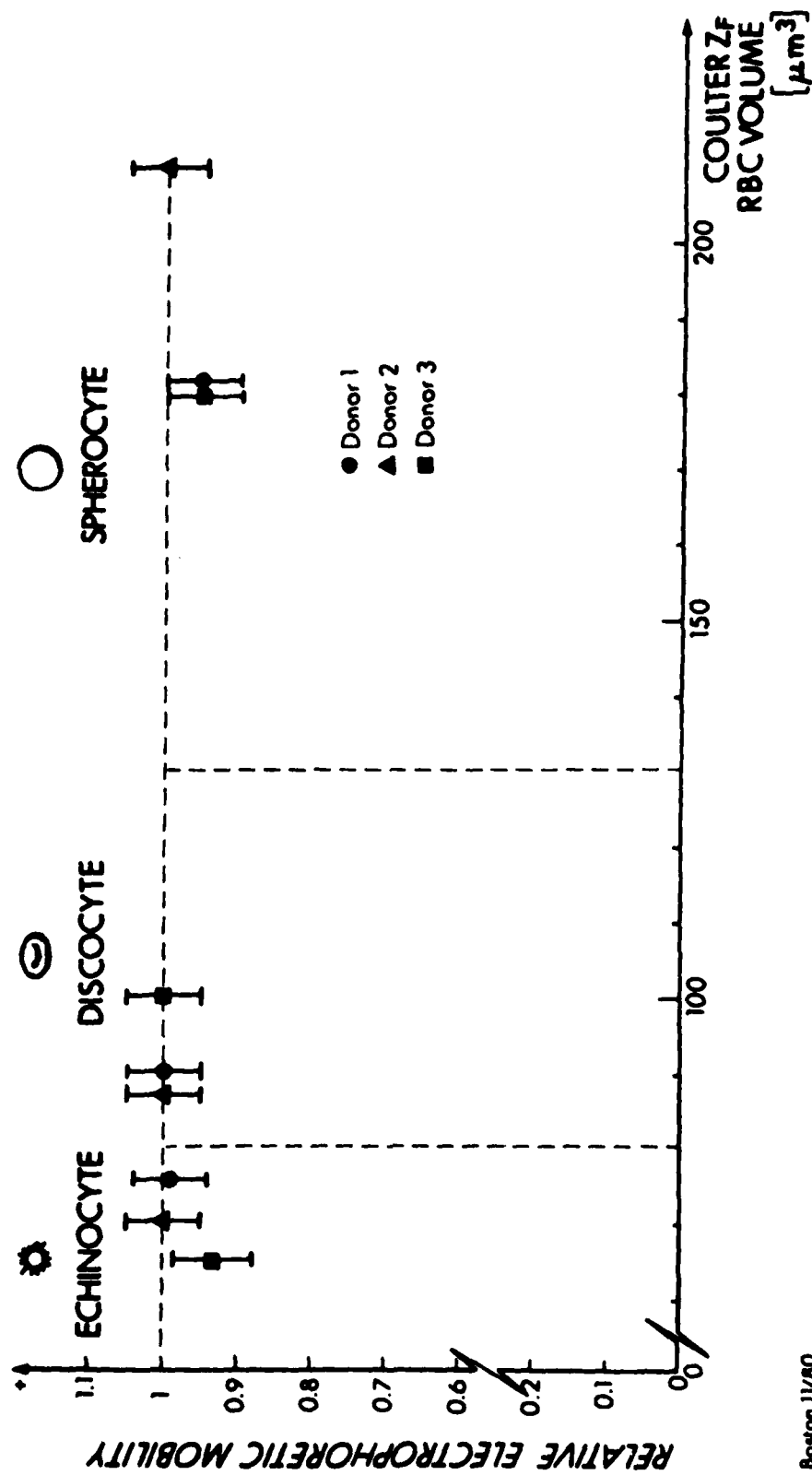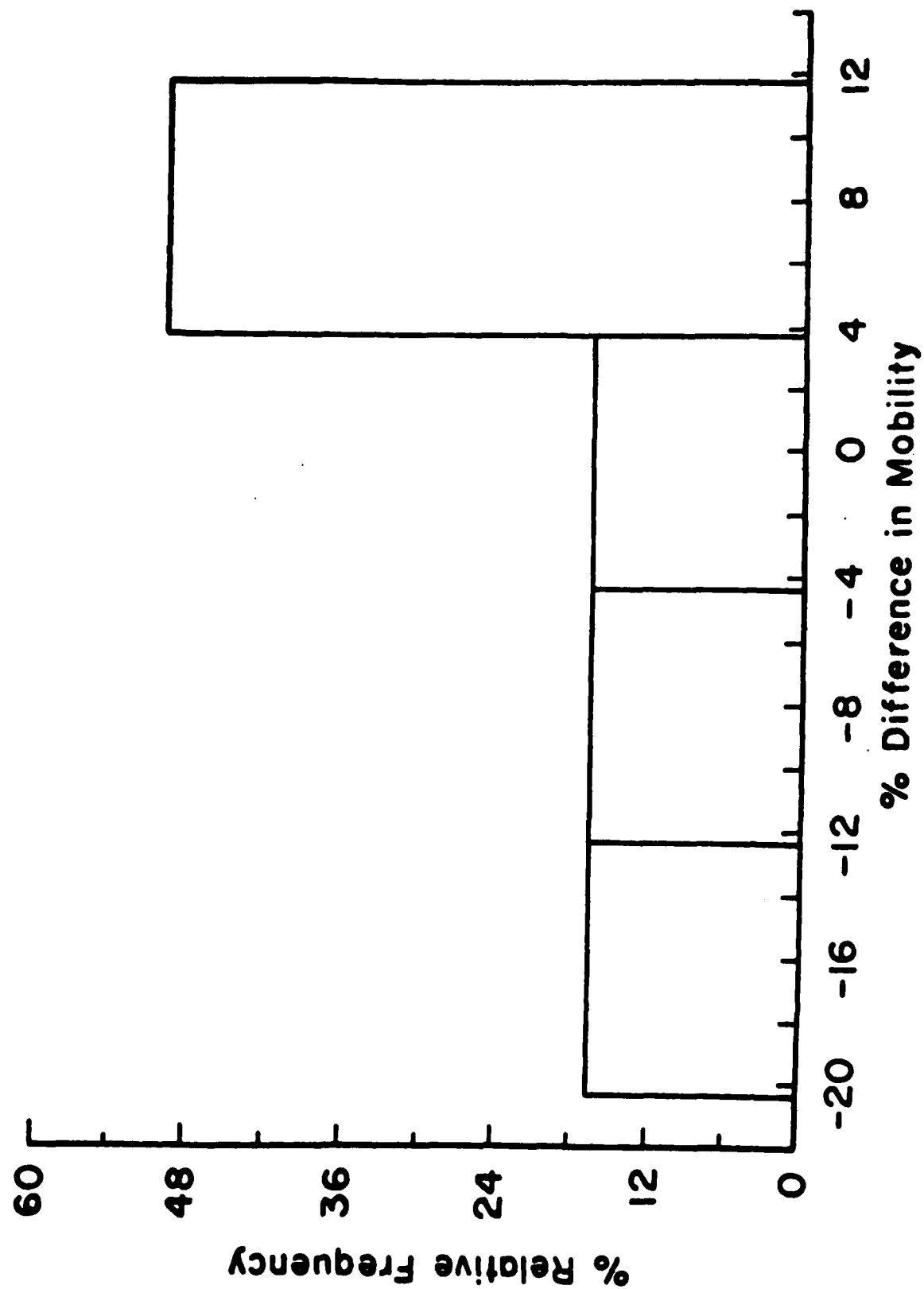and volume.

FIGURE 9

NBRL, Boston 11/80

FIGURE 10

Histogram of percent differences in electrophoretic mobility of

normal and Zymosan A activated human granulocytes isolated from blood

by two different methods.

FIGURE 10

% Difference in Mobility

% Relative Frequency

F. **APPENDICES**

APPENDIX I

Standard Operating Procedure for Electrophoretic Laser Light Scattering

The article "Apparatus and Methods for Laser Doppler Electro-phoresis" by Smith and Ware, Contemporary Topics in Analytical Chemistry, Vol. 2, edited by D. M. Hercules, describes the electro-phoretic light scattering (ELS) apparatus and its operation. The following provides the instructions needed for a technician at the Naval Blood Research Laboratory to operate our specific apparatus.

Coating Glass Windows:

The glass pieces are degreased using a gauze saturated with ethyl alcohol. Any spots that remain are flaked off with a scalpel. The glass pieces then are immersed in EtOH, and the vibrating portion of the Lab-Line ultratip Labsonic machine (4th floor) is placed in the alcohol. The amplitude is set at a position that gives a 40-60 power reading on the meter. The glass pieces are rinsed again with distilled water and are now ready to be soaked in

a 2% Z-6040 solution for 5 minutes. This solution is prepared by

combining: 16 ml MeOH, 4 ml H_2O, 0.5 ml Dow Corning Z-6040, and

1 drop of acetic acid (glacial). (This solution has a shelf-life of

60 minutes).

After soaking, the glass pieces are allowed to air dry and are

then baked in the Cryotherm (3rd floor) at approximately 140 F (setting

#5) for 2 hours. (Vacuum exhaust closed, gas supply closed, vacuum

breaker open, hours set at zero). Upon returning to room temperature

the glass pieces are soaked in a 0.1% methyl-cellulose solution for

5 minutes. This solution is prepared as follows: 1 g of methyl-

cellulose and 1000 g of distilled H_2O. (This solution is centrifuged

at 3000 rpm for 20 minutes at room temperature to remove particulate

matter. The solution can be stored indefinitely at 4^0C). After air

drying, the glass pieces are baked again for 2 hours at approximately

140 F. When this has been completed, they are stored in small beakers

covered with parafilm. To avoid contamination, the pieces are handled

by plastic hemostats throughout this process (from the initial cleaning

up to storage). The final product may appear spotted. This is permissable as long as the spotted region is not associated with the scattering region of the assembled chamber.

Coating Plastic Spacers:

The plastic spacers are degreased with EtOH. They are then sonicated for 10 minutes in EtOH, rinsed with distilled water, sonicated for 10 minutes in a 2% solution of sodium dodecyl sulfate, SDS (solution appears slightly soapy when shaken), and then rinsed in water again. The portion of the spacer which must be neutrally charged is soaked in 0.1% methylcellulose for 5 minutes and then air dried. Check for bubbles which may have remained on the surface of the plastic after soaking; they result in an irregular surface. Dry these spacers for 2 hours at 140 F. Store in a beaker covered with parafilm.

Coating Lifetime:

The lifetime of this coating is dependent upon both the amount of coating removed during the rinsing between samples and adherence of the sample to the coating, thus, decreasing the optical quality of the glass as well as the charge neutrality of the walls of the chamber.

Typically, the coating is repeated every 10-12 hours of operation.

Between periods of operation, filtered (0.45 μm Millex) distilled H_2O

is placed in the chamber.

Electrodes:

The electrode surfaces exposed in the sample chamber are 99.99%

pure Ag. They are cemented in place using Echolbond Solder 56C (Emerson

and Cuming, Inc., Canton, MA). The silver electrodes are cut with

scissors from a sheet of silver to the approximate dimensions and then

shaped to the proper curvature. They are cleansed in concentrated

NH_4OH, rinsed with water and cemented. They are then trimmed with a

sharp scalpel so they are flush with the rest of the electrode.

Anodization of the electrode surface is accomplished by polishing

the surface of the pure silver electrode with 4/0 3M-brand emory paper

and then cleaning it with concentrated NH_4OH. The two metal halves of

the chamber, one of which has been coated with stopcock grease, are

placed flush and screwed together. One alignment pin is inserted to

provide electrical contact between the halves. The provided plastic

"floor" is put in place and is sealed with more grease. The electrode

area, now defined by the plastic floor and the 2 clean silver electrodes,

is filled with 0.1 M HCl. A platinum electrode (from Coulter Counter)

is hooked up electrically to the negative pole of the constant current

power source. It is then placed so it dips into the 0.1 M HCl. A

second wire is attached, connecting the 2 halves of the chamber to the

positive pole of the power source. The current is turned to 5 amps,

the voltage is set on 20 volts. The power is turned on for 2.5 minutes,

after which the 0.1 M HCl is replaced with fresh, and the power is

turned on for 2.5 more minutes. This is repeated until the surface

of the electrodes is black. Disassemble the chamber and rinse the

electrode with distilled water. The surface of the electrode must not

be touched. If it is, clean it with EtOH. This anodization is repeated

every time the chamber plastic and glass parts are changed. The electrode

will not plate where there is grease so they must be very clean.

Chamber Assembly:

The clean portions of the chamber are assembled in the following

fashion: first choose 4 alignment pins that fit snugly in one of the halves of the electrode. Lightly grease the surface of this electrode where the plastic inserts will be mounted. (Avoid placing grease in the area of the groove in the plastic spacer). Place the plas.ic inserts in position (according to proper number, i.e., 22 22X and in an orientation directed by the number or design on the electrodes, i.e., 2 and 11). Slide the aligning pins up through the holes in the plastic inserts.

Grease the exposed surface of the insert in a fashion similar to that on the previous side. Place the second electrode into place (oriented correctly) and push the alignment pins into position. Now, screw the chamber snugly together and remove the alignment pins.

Choose 2 glass windows which have no optical obstructions in the area that will be associated with the gap area. They may be handled, but only at the periphery. Choose a third window of poor quality. All of the windows have been trimmed so they fit as flush to the assembled chamber as possible. This has been accomplished by beveling the windows

around the periphery. Look for this bevel because it should face the internal aspect of the chamber when assembled.

Using a syringe filled with stopcock grease and fitted with a cut #25 gauge needle, apply grease to the portion of the plastic insert which will approximate the glass window. For this initial fitting of the window, it is wise to keep the grease at least 1 mm from the gap area. Place the poor quality window in place and the grease will be extended. Lateral extrusion of the grease is of little consequence but if the grease gets into the gap the plastic parts have to be replaced. Often 2 or 3 replacements of this trial window are required to get the grease so it comes as close to the gap as possible without entering the gap. Do not force this window in place. The idea is for the perfect window to be pressed in place, thus achieving a good seal with the glass. The poor quality window is used to position the grease in a manner that will allow for a good quality seal between the glass and plastic parts yet will not result in excess grease being forced into the gap area when doing so.

When a layer of grease meeting these requirements is established, place more grease in the recessed circular portion of the chamber and put the perfect glass piece in place. Now place grease between the side of the glass window and the side of the most superficial circular portion of the chamber and press the teflon spacer in place. Finally, push the teflon window retainer (gasket in place) in position and tighten the screws. Excessive force applied by screwing the screws excessively may cause grease movements at gap area. Repeat this procedure on the other side.

The cross-sectional area of the gap is greatly influenced by the approximation of the grease to the edge of the plastic parts. Unfortunately, grease placement really does not let you be very precise. Therefore, it seems best to accept the fact that the cross-sectional area of the gap will change each time the chamber is assembled, so when manipulating the grease play it safe and keep it away from the gap. Grease in the gap may hinder the passage of the beam through the chamber or collect air bubbles and dust. The basic rule of thumb for chamber

assembly is to be conservative in your grease applications. Excess grease causes the above problems and will clog up the needle used to exchange samples from the chamber. However, enough grease must be used to avoid leaks. Leaks usually display themselves as a drop of water at the junction of the window and the teflon collar. The voltage display will show a decreased value (2 volts or less at 0.6 amps, 20 volts).

Constant Temperature H_2O Bath:

The constant temperature water bath hoses must be connected to the chamber. Obviously, one "out" flow and one "in" flow hose must be connected to each side. Wrap teflon tape around the connectors to help avoid leaks. These external leaks display the same symptoms as internal leaks.

The constant temperature water bath is located under the table. The flow control arm should be positioned to the maximum flow side. Both switches in front must be turned on. The solution in the bath is 50:50/glycerol:water. The bath temperature is controlled by the knob

labeled one through ten; the screwing up or down of this causes the

opening or closing of a mercury (thermometer) switch. Equilibration

of $\pm 10^\circ$C can be accomplished in as little as 20 minutes. When the

heater light is switching on and off intermittently, the bath is

equilibrated.

The temperature of the water bath should be the same as the samples

or slightly less. This minimizes bubble formation as well as decreases

the in-chamber equilibration time necessary before the measurement may

be initiated.

System Alignment:

The beam from the laser must have the same long axis as that of

the tube in front of the PMT (photomultiplier tube). Since any intense

light (including that from the room) could damage the PMT, the slit in

front of it must be closed during manipulation. This is accomplished

by unscrewing the micrometer to a position of 4.5. With a beam of such

intensity that is easy to visualize, adjust the beam so it passes through

the center of the closed adjustable diaphragm on the end of the tube and

lands on the pinhole (or slit) on the surface of the PMT while the tube

is set at 0°. Gross adjustments are accomplished by movement of the

mirror of the system. Fine tuning may be achieved by use of the lateral

adjustment under the diaphragm and the vertical adjustment under the

slit. The lens must be inserted at its proper focal length (20 cm from

the gap area) and its insertion cannot affect the path of the beam to

the slit. Any density filters placed in the system are placed perpen-

dicular to the beam. This is accomplished by aligning the filter so

the laser beam's back reflection retraces its path.

Any lens or filter that the beam travels through must have a very

rigid connection to its stand, which in turn is firmly attached to the

optic table. Any optic equipment which is placed in a lens holder

should have a washer of a soft material (rubber or a rolled loop of

teflon tape) to help strengthen the grasp of the holder on the lens.

Filters require a portion of clay (such as found in the "Sealocrit")

to be placed as a vibration cushion at each corner. One must realize

that vibrations on the order of even a portion of the wavelength of

light can cause problems in the signal quality. The adjustable

diaphragm on the tube in front of the PMT is not used for this very

reason. A metal plate with a hole drilled in it is mounted over the

wide open diaphragm.

Positioning of the Chamber:

The chamber is mounted on the plastic bracket on top of the micro-

meter. The bottom of the chamber is coated with grease to prevent

problems associated with liquid spillage and is held in place by 4

allen screws.

Orientation of the chamber is such that the needle insert holes

are on top and the orientation marks on the chamber face the PMT. The

hoses from the constant temperature bath must not be impinged.

The chamber is adjusted via the compass on the bottom of the micro-

meter so that the window closest to the laser is perpendicular to the

laser beam. This is done by checking the back reflections.

The beam must be at a level 1/2 way between the plastic inserts.

Adjust the vertical micrometer so that the first sign of the beam hitting

one plastic spacer is observed. Record the micrometer reading. Screw

the micrometer until the same light is observed on the other plastic

insert. Subtract the two values and reposition the micrometer to the

1/2 way point. Lateral positioning is of less consequence since the

entire gap has a uniform electrical field. Measurements may be made

anywhere in the center 3/4 of the gap on the horizontal axis.

If any of the previously mentioned components are loose, the

result is one or more spurious peaks in the power spectra. These peaks,

unlike peaks resultant from electrophoresis, will not shift or disappear

when the voltage is altered.

The alignment of the system can take many hours (depending on how

complete the system was to start). Alignment can be checked periodically

if you mark on the wall the position that the beam will hit when the PMT

is rotated out of the way. Realignment is seldom necessary. The

system is basically set up now. A final adjustment will be required

when the sample measurements are made.

Medium Selection

Measurements are made in iso-osmotic sucrose buffer solution of pH 7.4 and ionic strength of 0.1, made by diluting one part PBS with 14 parts 0.29 molar sucrose (49.64 g.liter distilled H_2O). The solution is stored at $4^{\circ}C$. Hours prior to measurement, the buffer is filtered (0.45 μ) and premeasured. This allows for removal of particulate impurities, elimination of bubbles, and temperature equilibration.

The ionic strength of the medium can influence the data. When selecting an ionic strength one must keep in mind the biology of the system and be aware that low ionic strength mediums may have a detrimental effect on the sample.

All ionic strengths will show different technical peculiarities. Educated trial and error changes, based on information placed in this paper concerning each aspect of the apparatus, will be the best guide to correcting technical problems.

Laser Intensity:

A laser intensity must be selected that is low enough to produce minimal heating of the sample volume and minimal damage to the sample, yet is high enough to produce adequate intensity for analysis with a high enough signal-to-noise ratio for adequate interpretation.

The current produced by the PMT shows pulses that correspond to pulses of light scattered by the cells (see accompanying print-out). By increasing the intensity it may be possible to make these pulses 5-10 times larger than the peaks in the background signal. This is optimal. Typically, using the 1 watt scale, a current reading of 4-6 on the laser control box (40 m watt) will produce an adequate intensity.

Collection Time:

The minimum duration of a single pulse of current is determined by the special resolution necessary for the measurement. Samples which display greater Doppler shifts can be measured with shorter signal collection times. This shift may be increased by increasing the velocity of the particle (increase the amperage or decrease the ionic strength

of the medium) or by increasing the angle at which the scattered light
is collected.

The collection periods are separated by delays which allow for
the dissipation of heat produced during the pulse. Factors present
in establishing this delay period are the heat dissipation time constant
of the chamber, conductivity of the medium, current, laser intensity,
and collection time length.

Collection delay periods are repeated until a significant number
of cells have been sampled. A 10-second collection with a 5-second
delay repeated 4 times has proven sufficient for our RBC samples using
our listed parameters.

Collection Angle:

As the angle of the PMT is increased, two things occur. First,
the intensity of the scattered light decreases. Secondly, the detected
Doppler shift becomes greater. Therefore, one typically tries to obtain
a shift which is easy (rapid) to measure in terms of collection time
(collection time inversely related to spectral resolution), yet still

allows for the use of a laser intensity that is low enough so not to

damage the cells exposed to it. Trial and error prove to be the best

way of determining this. We have used an angle of 10°.

Sample Concentration:

The sample concentration must be established on two criteria. The

sedimentation rate of the sample must be evaluated. A concentration is

then chosen which will allow for a significant number of particles to

pass across the beam throughout the entire collection process before

significant sedimentation had occurred. This is evaluated by use of the

photomultiplier current output. Both the first and last collection in-

puts should have similar number of signal pulses. The operator looks

for 5-12 pulses of photo current to appear during one cycle of the

collection time.

The power spectra of the sample must also be evaluated. High

concentrations produce multiple scattering which results in a broad

low frequency peak and also broadens the shifted peak (see Example 3).

Erythrocytes:

Erythrocyte samples (either whole blood or packed cells) are stored at 4°C until prepared for measurement. The cells are initially in the iso-osmotic sucrose buffer solution to an estimated concentration of 1200-7000 cells/λ. The precise concentration of the suspension and the MCV value of the RBC's are then measured by a Coulter Analyzer. Final adjustment of the RBC concentration to 60 cells/λ is accomplished by making a second dilution with the iso-osmotic buffer.

Platelets:

Platelets are prepared in an identical fashion to the erythrocytes. The final concentration of the suspension should measure 200 cells/λ.

White Blood Cells:

White blood cells are prepared to a concentration of 60 cells/λ. It is easiest to pre-measure iso-osmotic buffer into aliquots (such as 5 ml) and make dilutions via usage of an adjustable micropipet.

Standard:

A RBC suspension in iso-osmotic sucrose buffer solution containing

1% glutaraldehyde (stored at $4^{\circ}C$) is used as a standard. Day-to-day fluctuations occur due to changes in the cross-sectional area of the gap, electrode resistance, buffer conductivity, and electro-osmosis. The standard is run prior to and in between sample runs so as to provide a reference point for comparison. When comparing data from different days of operation, the electrophoretic mobility of the sample is divided by the electrophoretic mobility of the standard.

Pre-measurement alignment of the system is performed with the standard red cell solution as a sample. The mode of the spectrum of this sample will vary from day-to-day as previously discussed, but the velocity profile of the red blood cells is very narrow and reproducible.

With the laser properly positioned in the gap containing the RBC standard, the laser is moved horizontally until a speck imperfection in the coating of the inside wall of the glass window closest to the PMT is detected. Imperfections show as bright points of light on the glass surface because they cause light to be scattered intensely. The

result is a bright image of light at the slit on the PMT. This light

acts as a reference beam. This light does not undergo a Doppler shift

because it is scattering from a non-moving object. Because it is unshifted

it is used as a standard to which the incident beam (shifted light

scattered from the moving RBC's) is compared. This is the whole basis

of the technology. Once this reference beam effect has been obtained

at the slit measurements may be initiated.

From start to finish the procedure currently being followed for

RBC and WBC samples is as follows:

1. Turn water source for laser on. Watch pressure build up

   to 30-40 PSI.

2. Turn master control on laser to "on" position.

3. Pull up on the 4 "line switches" on the laser control box.

4. Wait until the "ready light" lights up and press the "start

   button".

5. Adjust the current value to 4. (The laser is now on).

6. Turn on constant water bath and allow to equilibrate (10-15

   min) and record the temperature.

7. a) Turn on amplifier (on-off push button on lower right).

   b) Check to see if input A comes from the PMT.

   c) Output must go to "signal in" on FFT.

   d) Do a battery test (see toggle on front of amplifier).

   e) Roll-off = 0.1, gain = 2k, hi roll-off = 30 k, input A = DC.

8. Turn on volt/amp meter and check to see if associated box is

   set on volts/amp: amp = 2m scale; volt = 200 volt scale.

9. Turn on FFI (lower left corner). See that is is set accordingly:

   slope = Ext

   run = Ext

   trigger/level = 0 and "multiply"

   signal in = AC

   weighting = flat

   overload = adjust so overload light is not on

   time window = 10 sec

   clock = internal

   gain = auto

   scope = scope

   count = 16

   lower right toggle = off

10. Turn on oscilloscope (lower right pull out).

11. Turn timer panel on (lower right corner). Set to: "Auto", "off", delays = 5 and 1. Always press the "reset" button when turning on the apparatus.

12. Turn on power source (lower left).
    voltage = about 20 volts
    range = 1 mAmp
    current = 0.600 mAmp

13. Turn on voltage supplt to PMT (toggle and push button).

14. Turn on interface (push button with associated light).

15. Go to 2nd Floor:

    A. Turn on interface #2 (by sink).

    B. Turn on HP computer (back right side).

    C. Turn on all floppy disc mass storage devices (front right).

    D. Insert a disc containing the program labeled "ELS" into mass storage device F8,0.

    E. Type in Get "ELS:F8,0" and press the execute button.

    F. In response to the question "Number of last measurement?" you can write in any number you desire as long as the number has not already been used to identify another recorded spectrum on the floppy disc. (This number should be recorded

because all transferred spectra will be numbered serially

from this point and thus serves as a method of identification).

The apparatus is now on and is ready to make measurements. No warm-up

other than for the water bath is necessary.

During periods when the computer is being utilized by other projects

the ELS apparatus may be turned on in the following manner:

A. Turn on interface #2.

B. Turn on H/P computer.

C. Type in "WRITE BIN 2;0,4"

D. Press EXECUTE

The computer may not be used for data storage in this mode.

Pre-Sample Preparation:

Three buffer solution rinses are introduced into the chamber via

a 23-gauge needle, following which a sample of the standard is inserted.

The scattering volume of the apparatus is then checked with a low power

optic for air bubbles or debris which may have lodged in the gap area

at this time. A measurement of the standard is then initiated (see

following discussion) during which the chamber is moved horizontally

across the beam until proper optical mixing has been achieved as

indicated by the quality of the PMT current signal (Examples 1 and 2)

and the resulting power spectrum (Example 3).

Once the proper mixing has been achieved, the samples are run in

the following fashion: The sample is inserted into the chamber and the

laser beam is unblocked (the beam is always kept blocked between runs

for safety reasons as well as to protect the sample). The current

pulse/collection time is initiated by pressing the restart button on

the timer panel. As soon as the "cell temp" light has gone off (this

lamp indicates that the FFT and timer are synchronized and are both

ready to begin a collection cycle), the toggle on the lower right of

the FFT is turned from the "off" position to the "start" position.

Immediately press the "start" button on the timer panel to begin the

collection (visualized by the input on the cathode-ray tube or by the

polarity indicator on the timer panel switching from "off" to either

"positive" or "negative"). The collection cycle's end is indicated

by the finish light on the FFT. It stops flashing and stays on. The

laser beam should be blocked.

The electronics of the system have now computed the power spectrum

of the individual pulse/collection cycles and has averaged these

successive spectra into the spectrum displayed by the graphics plotter

or recorded on a floppy disc by the H/P computer which is interfaced

with the electronics of the ELS apparatus (Examples 1, 2, 3).

The plotter is operated in the following manner. The "power"

switch on the plotter should be turned on and a piece of graph paper

should be put in place and the "chart" button depressed so to hold the

paper. The "servo" motor switch should be turned on. The switches

in the middle right portion of the FFT should be set to "plot" and

"cal 0.0". The "marker" switch is then turned to the "off" position.

The red "push to start" button is pressed and plotting begins. When

plotting is finished, return the "plot" toggle to the "scope" position.

The mode of the resulting spectra must be recorded via use of the

toggles mounted in the center of the FFT. The toggle must be in the

"off" position for the data to be plotted.

Recording the spectrum directly onto a floppy disc by the computer is a much more accurate and rapid transfer of information. Once the collection cycle has ended, the red transmit button on the panel below the FFT is pressed. The button is depressed until the yellow light below it flashes. This light indicates that the information has been sent. It takes about a minute for the computer to accept all of this data. During this time it is important not to repress the button or reset the ELS electronics. The transferred data is recorded on the floppy disc and is numbered as "EXXXX".

The voltage and amperage must be recorded for each sample. See the accompanying sheet that has been used to characterize samples.

The sample is now removed. Repeat the above procedure starting with the three rinses of plain buffer solution. Turning off the apparatus is a straight reversal of turning it on. Distilled water must be placed in the chamber until the next time it is used.

APPENDIX II

Standard Operating Procedure for Laser Velocimetry

Particle velocity can be measured by use of laser principles other than the Doppler shift. One such method is based on the ability of a particle to reflect an interference pattern produced by two crossing laser beams.

Apparatus Assembly:

The optical set-up for this technique is extremely simple (Example 4). The laser beam is divided into two beams by a cube beam splitter that has an angulation which results in the crossing of the two emerging beams. These beams produce an interference pattern when they cross due to their wave-like nature (as the beams pass in and out of phase with each other they produce areas of brightness and darkness). The beam splitter does not divide the beams into beams of equal intensity; in order to increase the signal-to-noise ratio, the beams are equilibrated by inserting the corner of a density filter in the path of the brighter beam somewhere in the region between the cube and where the beams cross.

The PMT could be used for such an adjustment, but the eye proves to be a satisfactory judge of when the brigher beam has been properly attenuated.

A chamber containing the moving particles of interest is mounted so that the particles to be measured pass through the region where the beams cross. As the particles pass, the interference pattern produced by the beams is reflected to the PMT. Here the electronics count the number of reflected patterns reflected by the particles per collection period. The signal-to-noise ratio of the system is increased by positioning the PMT so that it bisects the angle formed by the crossing beams, thus the PMT receives equal amounts of light from each beam.

The particles must have a length that is less than the width of one band of the interference pattern. When working with particles of a fixed size (i.e., RBC's), the band width may be altered by changing angle 0 (the angle of the crossed beams). The greater angle 0, the smaller the overall size of the interference pattern, thus the smaller sampling area. The width of the bands may be calculated by:

$$\delta = \lambda/2 \ \sin\beta$$

Operating Instructions:

The apparatus is turned on in the following manner:

1. Turn on water source for laser. Watch pressure build to 30-40 PSI.

2. Turn master control on laser to "on" position.

3. Pull up on the four "line switches" on the laser control box.

4. Wait until the "ready light" lights up and press the "start" button.

5. Adjust the current valve to "4" (the laser is now on).

6. a. Turn on amplifier (on-off push button on lower ritht).

    b. Check to see if input "A" comes from the PMT.

    c. "Out put" must go to "signal in" on FFT.

    d. Do a battery test (toggle on front of amplifier).

7. Turn on FFT (lower left corner). See that it is set accordingly:

    a. slope = EXT

    b. run = INT

    c. trigger level = "0" and "multiple"

    d. signal in = AC

    e. weighting = Flat

    f. overload = adjust so overload light is not on

    g. clock = internal

    h. gain = auto

    i. scope = scope

    j. lower right toggle = off

8. Turn on oscilloscope (lower right pull out).

9. Turn on voltage supply to PMT (toggle and push button).

10. Turn on computer interface.

11. Go to 2nd floor.

    a. Turn on interface #2 (by sink).

    b. Turn on H/P computer (back right side).

    c. Turn on all floppy disc mass storage devices (front right).

    d. Insert a disc containing the program labeled "ELS" into mass storage device F8,0.

    e. Type in - Get "ELS:F8,0" - and press the execute button.

    f. In response to the question "Number of last measurement?", you can write in any number you desire as long as the number has not already been used to identify another recorded spectrum on the floppy disc. (This number should be recorded because all transferred spectra will be numbered serially from this point and thus serves as a method of identification).

The apparatus is now on and is ready to make measurements.

During periods when the computer is being utilized for other projects, the apparatus may be turned on in the following manner:

1. Turn on interface #2.

2. Turn on H/P computer.

3. Type in - WRITE BIN 2;0,4-.

4. Press EXECUTE

The computer may not be used for data storage in this mode.

EXAMPLE 1. Sample output of the electrophoresis signal versus time. NOTE: there is good discrimination between the background signal and the platelet signal.

EXAMPLE 2. Sample output of the electrophoresis signal versus time. NOTE: There is inadequate discrimination between the background signal and the platelet signal.
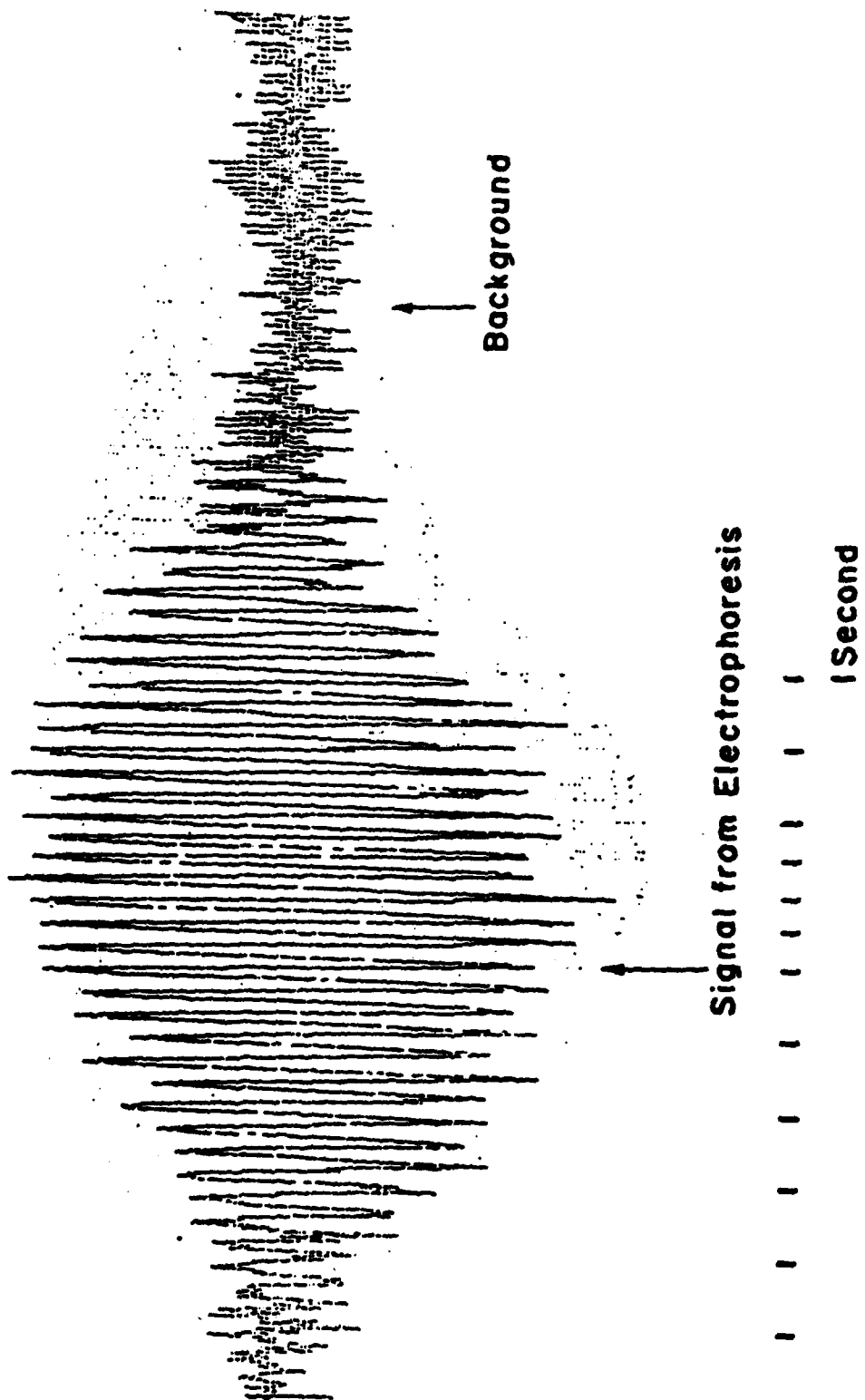
EXAMPLE 3. Sample output of the power spectrum versus frequency for a red cell sample.

EXAMPLE 4. Block diagram of the apparatus for laser velocimetry.
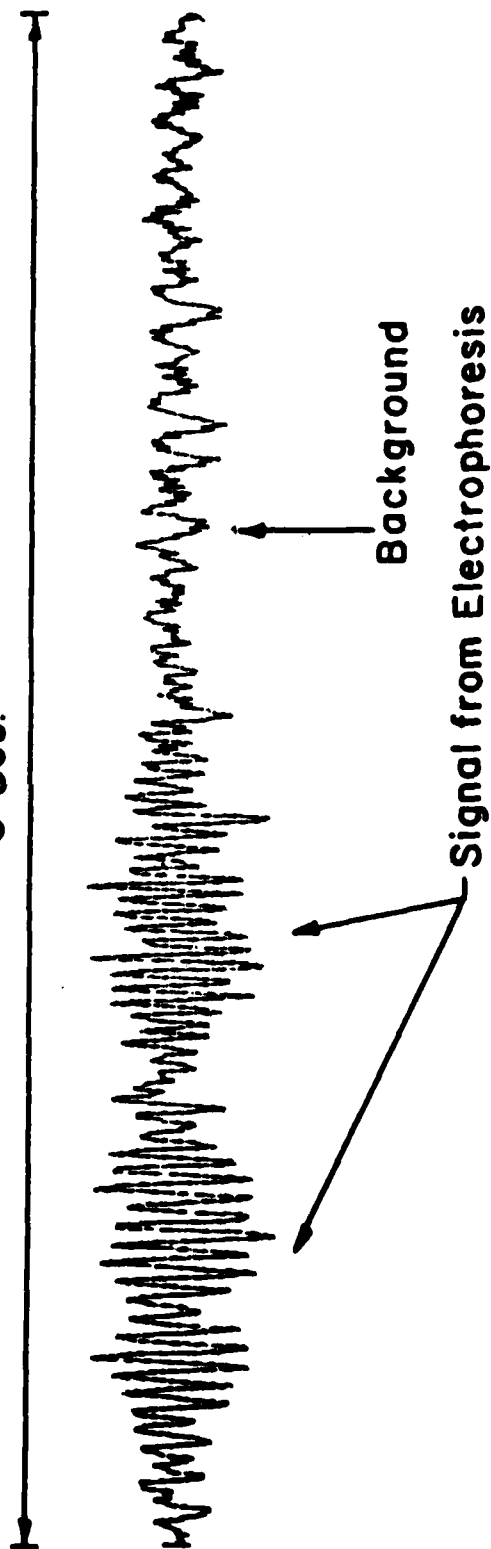
EXAMPLE 1

Platelets

Good Signal to Noise

Background

Signal from Electrophoresis

1 Second

EXAMPLE 2

Platelet

Conc. = 30 Cell/λ

Poor Signal to Noise

5 Sec.

Background
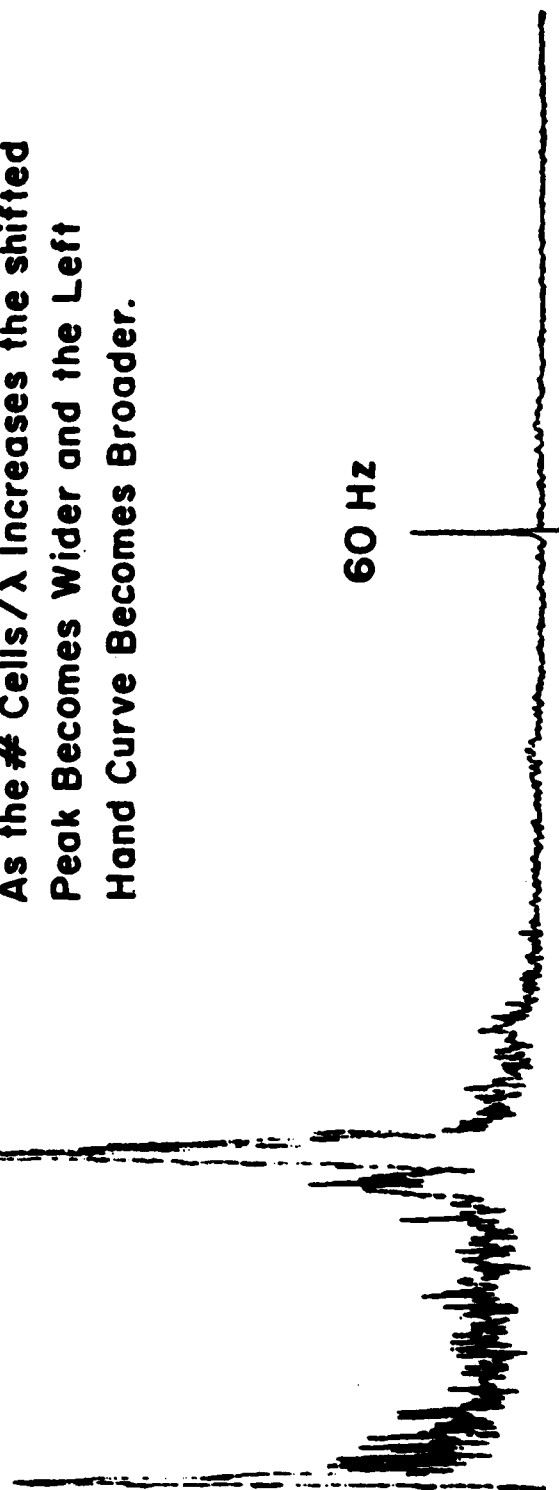
Signal from Electrophoresis
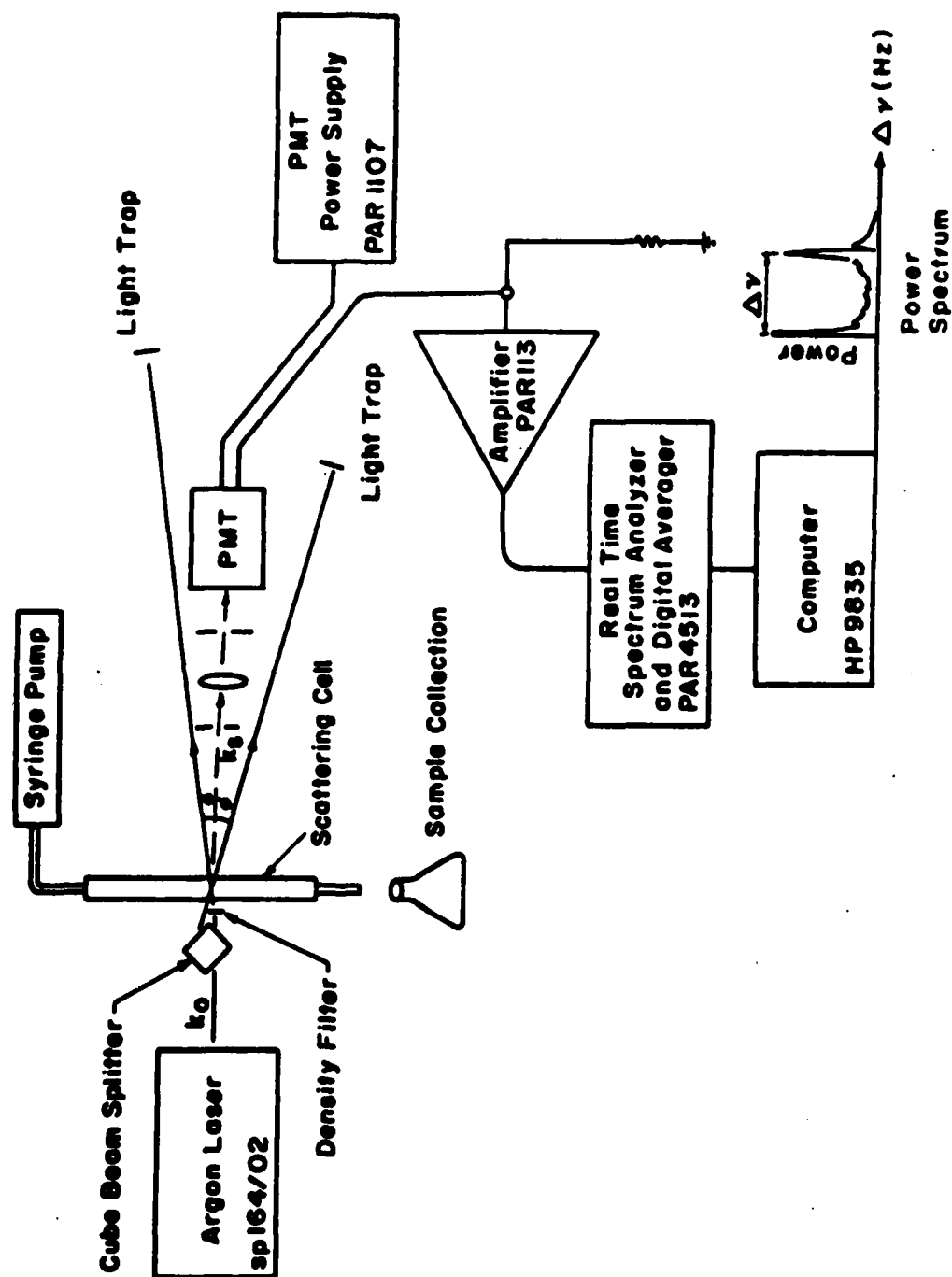
EXAMPLE 3

Red Blood Cells

Optimal Power Spectra

Peak Resultant of Electrophoresis

As the # Cells/λ Increases the shifted
Peak Becomes Wider and the Left
Hand Curve Becomes Broader.

20.8 Hz

60 Hz

EXAMPLE 4

F. <u>References</u>

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